

Fibrinogen Preparations for Tissue Engineering Approaches

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Introduction

One of the key issues in tissue engineering is the use of an adequate scaffold material for seeding procedures with distinct cell types [1]. Fibrin matrices have been found to be an appropriate scaffold material for the seeding with vascular cells in cardiovascular tissue engineering [2]. The matrix can be invaded by different cell types such as fibroblasts and smooth muscle cells and provides a space for the three-dimensional cellular arrangement [3,4]. During a remodeling process the fibrin matrix is replaced by a scaffold of newly synthesized extracellular matrix proteins [5]. For tissue engineering approaches sufficient mechanical properties in the early stages of seeding and remodeling processes and the ability of spreading and proliferation of seeded cells are the major demands on the matrices.

Fibrinogen plays a key role in various processes, such as haemostasis, thrombosis, platelet adhesion and aggregation and serves as temporary matrix for cell in growth and proliferation during wound healing [6]. Separation and enrichment of fibrinogen is required to obtain a fibrin matrix suitable for seeding procedures. A variety of methods have been described for the separation of clottable protein/fibrinogen from plasma [7-14].

The objective of this study was to compare three different methods for the separation of a fibrinogen preparation from peripheral blood with regard to the quantity of the separated fibrinogen/ protein precipitation and their ability for the seeding with vascular cells isolated from peripheral blood, and their mechanical properties.

Materials and Methods

100 ml blood (n=10) were drawn under sterile conditions from pig's (female, Deutsche Landrasse) internal jugular vein and collected in heparinised syringes (100 units heparin per ml, Braun, Germany). The plasma and the monocyte fraction were separated by centrifugation at 900 g and 20°C for 12 min. For isolation of late outgrowth smooth muscle cells (LOSMC) the monocyte fraction was aspirated and resuspended in phosphate buffered solution (PBS, Lonza, Switzerland). The monocyte fraction was washed twice in PBS and resuspended in Smooth muscle basal medium + supplements (SGM, Lonza). Each suspension was given into gelatine-precoated (1% bovine gelatine; Sigma-Aldrich, Germany) culture flasks (Nunc Nalgene, Denmark). Medium was changed every third day. After grown to confluence cells were detached with trypsin (Lonza) and expanded 1:3.

Fibrinogen separation

For separation of fibrinogen the plasma was declined and separated into three equal parts for the precipitation of the fibrinogen by three different methods:

- cryoprecipitation
- ethanol-precipitation
- ammonium sulphate (AS) -precipitation.

Cryoprecipitation

The plasma was stored at -20°C for 24 hours, thawed at room

temperature and centrifuged at 600 g for 3 min. The supernatant was decanted and the remaining hydrous pellet containing fibrinogen and other plasma proteins dissolved completely at 37°C. A protein-gel was obtained.

Ethanol precipitation

The plasma was cooled to 4°C before ethanol (70 vol. %, 4°C) was added in a ratio of 4:1 (plasma/ethanol). Immediately afterwards the plasma/ethanol-solution was centrifuged at 600 g at 4°C for 3 minutes. The supernatant plasma was decanted and the fibrinogen pellet dissolved at 37°C. Prior separated plasma and aprotinin-solution (10.000 KIU per mL, Bayer, Germany) were added (0-2 mL) until the pellet was completely dissolved.

AS-precipitation

The plasma was cooled to 4°C and a saturated solution of AS (Sigma-Aldrich) in phosphate buffered solution (PBS, Lonza) without calcium and magnesium was added in a ratio of 3:1 (plasma/ AS-solution). The remaining steps were the same as described for the ethanol precipitation.

Quantification of protein and fibrinogen in the precipitation

The amount of total protein was calculated by measuring the dry weight of the precipitation. The concentration of fibrinogen was measured by means of an enzyme-linked immunoassay (ELISA) (Innovative Research, Michigan, USA) according to the manufacturer's instructions. In brief in this assay the fibrinogen reacts with antibodies against fibrinogen adsorbed to the surface of polystyrene wells conjugated with horseradish peroxidase, which were stained by addition of 3,3',5,5'-tetramethylbenzidine. Its quantity directly correlates with the concentration of fibrinogen in the sample and with the absorbance which was measured at 450 nm in a microplate reader. The concentration of fibrinogen was calculated from a previously established standard curve.

Generation of fibrin matrices

For the generation of a fibrin matrix 1 mL of fibrinogen precipitation was mixed in equal parts with a thrombin-solution (500 µl calcium chloride-solution (40 mMol/L) (Sigma-Aldrich), 200 µl aprotinin-solution (10.000 KIU/ mL), 200 µl protamin (5000 units/ mL; Valeant, Germany) and 20 units thrombin (Vascular Solutions,

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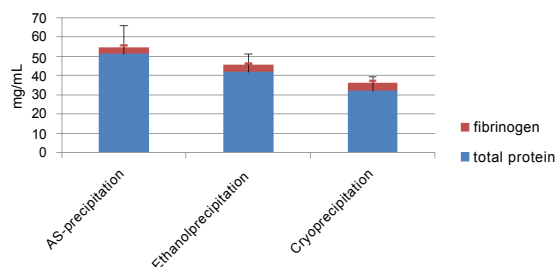
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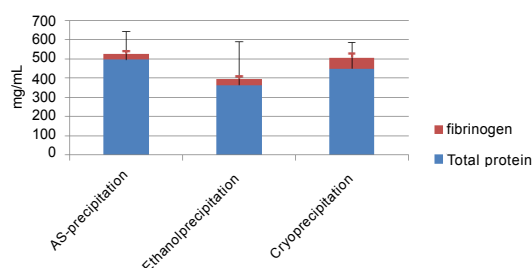
Figure 1: Experimental setting for the mechanical testing of the generated matrices. Matrices were connected via clamps to a device measuring the force occurring by the ends are pulled apart until the matrix bursts.

Amount of protein and fibrinogen separated from plasma



a)

Concentration of protein and fibrinogen in precipitation



b)

Table 1: By means of addition of chemical compounds the greatest amount of protein and fibrinogen was separated from heparinized plasma (a). Separated protein dissolved easier when separated by means of cryoprecipitation resulting in significant higher concentration in the precipitation obtained by cryoprecipitation (b).

Minnesota, USA)). Both components were given under simultaneous stirring via a Y-connector (Baxter, Germany) into chamber slides (Nunc Nalgene). Fibrin matrices with a size of 1.8 x 0.8 cm and 0.2 cm thick were obtained. Seeding procedures were performed in a density of 10^5 per cm^2 with cells of the fourth passage. Seeded and not seeded were incubated at 37°C and 5 vol. % CO_2 for 5 days. SGM-2 with 5% aprotinin-solution was added and changed every second day. In a second group 0.4 units factor XIII was added to thrombin-solution.

Characterization of the fibrin matrices

For mechanical testing the fibrin matrices were placed in clamps of a gauge, which measured length and tensile force (ATP Messtechnik, Germany) (Figure 1). The tensile strength was determined by pulling apart the construct and measuring the force required to rupture the matrix. Stress-strain was recorded while the sample was strained at 20 mm/min. Tensile strength was taken as the maximum stress sustained.

For visualization of the structure of the fibrin thin fibrin layers were gently squeezed and viewed under phase contrast microscope (Axio Observer, Zeiss, Germany).

The metabolic rate of the seeded cells was determined using the assay WST-1 (Roche Applied Science, Germany) which utilizes the cleavage of a tetrazolium salt by intracellular mitochondrial dehydrogenases to a formazan salt accumulating in supernatant. For this assay the medium was replaced with 300 μl fresh SGM-2 with 10% of the WST-1-solution. After an incubation time of 4 hours at 37°C and 5 vol. % CO_2 formazan concentration in supernatant was quantified via measuring absorbance in a microplate reader at 450 nm wave length. The results are shown calculated in relation to the initial metabolic rate of LOSMC seeded in a chamber slide (glass) which was set to 1.

Fluorescence staining

Isolated LOEC were characterized by immunofluorescence staining against CD31 (Acris, Germany). A secondary antibody linked to Cy3 (Dako, Denmark) was used. LOSMC were characterized by immunofluorescence staining against alpha-actin (Imgenex, USA) and smoothelin (Acris, Germany). A secondary antibody linked to FITC (Acris) was used. Cells and samples of seeded matrices were fixed in methanol/acetone (1:1) at -20°C . Samples of the seeded matrix were

Maximum tensile strenght of matrices engineered from protein/fibrinogen separated from heparinised blood

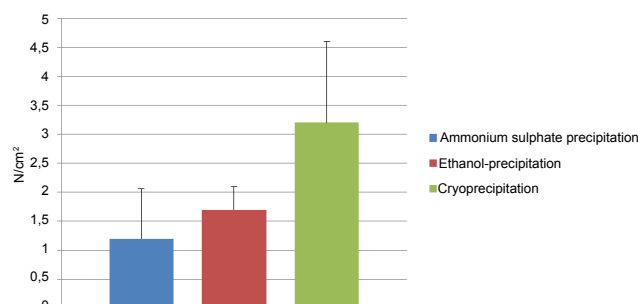


Table 2: Mechanical properties of the matrices generated from clottable protein. Significantly best tensile strength was measured in matrices generated from protein/ fibrinogen separated by means of cryoprecipitation. The mechanical properties of the matrices engineered from protein/ fibrinogen separated by addition of ethanol or AS were inferior.

stained using with 4',6-Diamidin-2-phenylindol (DAPI) visualizing the arrangement of the cells inside the matrix.

Statistical analysis

All statistical analyses of this study were performed with student's t-test in Excel 2007 (Microsoft). Mean and standard deviation (SD) were calculated for all continuous variables. The significance of differences between groups was determined on basis for normal distribution. Differences were considered as significant at $p \leq 0,05$.

Results

Protein pellets

A clottable protein fraction containing fibrinogen was successfully isolated by every of the three methods. The greatest amount of clottable protein was separated by addition of AS (48.6 ± 19.2 mg/ mL plasma). The least amount was obtained by means of cryoprecipitation (24.3 ± 7.9 mg/ mL plasma); ethanol-precipitation (31.6 ± 13.8 mg/ mL plasma). The amount of fibrinogen separated from plasma was highest by means of AS (3.2 ± 1.9 mg/ mL plasma) and lowest by means of cryoprecipitation (2.5 ± 0.9); ethanol precipitation (2.7 ± 1.7) (Table 1).

The obtained protein pellets dissolved with varying degrees. Whereas cryoprecipitated protein pellets dissolved easily, for the dissolution of the pellets of the AS- and the ethanol-precipitation titrated addition of 0-2 mL heparinised plasma and aprotinin-solution (ratio 1:1) was needed up to an addition of nearly 50% of the volume of final preparation (ethanol-precipitation: $46.6 \pm 8\%$, AS-precipitation: $49.6 \pm 17.4\%$). Namely in ethanol-precipitated protein preparations flocculation and premature clotting occurred. Different solubility

resulted in different fibrinogen concentrations in the final preparation, which was significantly highest in cryoprecipitation (total protein: 545.9 ± 105.3 mg/ mL and fibrinogen: 56.1 ± 20.8 mg/ mL and lowest in ethanol-precipitation (total protein: 240.3 ± 59.4 mg/ mL, fibrinogen: 18.6 ± 11.6); AS-precipitation (total protein: 317.5 ± 135.1 mg/ mL, fibrinogen: 24.0 ± 13.4 mg/ mL) (Table 1).

Tensile strength

The maximum tensile strength was 3.2 ± 1.4 N/ cm^2 of the matrices generated from cryoprecipitated protein/ fibrinogen, 1.7 ± 0.4 N of the matrices from ethanol-precipitated protein/ fibrinogen and 1.2 ± 0.8 N of the matrices generated from protein/ fibrinogen separated by means of AS (Table 2). Addition of factor XIII did not increase the mechanical properties of the matrices.

Whereas, phase contrast microscopy revealed a complex texture with a network of fibrils of the fibrin matrix generated from cryoprecipitated fibrinogen. Matrices generated from fibrinogen precipitated by ethanol and AS showed a less complex texture without visible fibrils (Figure 2). Addition of factor XIII to the thrombin component did not result in a more complex structure of the fibrin matrices generated from ethanol-respectively ammonium sulphate-precipitated protein.

Cell culture and seeding

By means of density gradient centrifugation and incubation with selective culture medium colonies of either LOEC or LOSMC were obtained. 7-10 days after isolation colony-forming cells entered the stage. Cells were expanded *in vitro*. LOEC had typical endothelial cobblestone-morphology and were positive for CD31 and eNO-

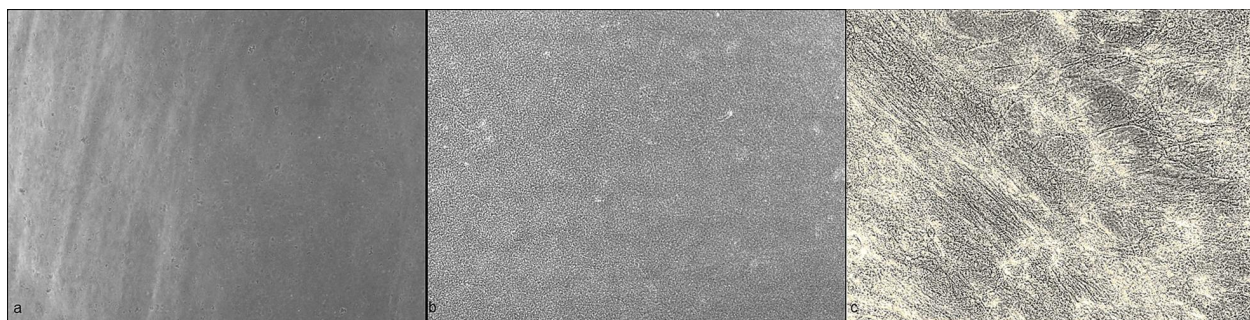


Figure 2: Phase contrast microscopy revealed a complex structure of the matrices generated from cryoprecipitated protein/ fibrinogen (c), whereas the matrices generated from protein/ fibrinogen separated by means of AS (a) or ethanol (b) had a significant less structured texture.

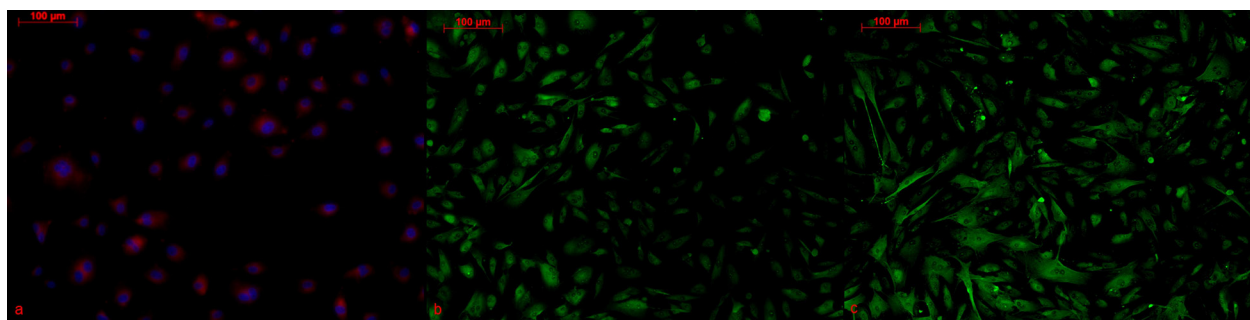


Figure 3: LOEC (above) and LOSMC (below) were isolated from peripheral blood by means of gradient density centrifugation. LOEC showed characteristics of endothelial cells and were positive for CD31, whereas LOSMC showed characteristic morphology of smooth muscle cells and expressed typical antigen sm- α -actin.

Synthase. LOSMC were identified by distinct characteristic morphology and antigens such as sm- α -actin and smoothelin (see Figure 3).

Proliferation assay WST-1

LOSMC seeded on matrices generated from ethanol-precipitated fibrinogen had the highest metabolic rate from day two on, which was most stable over the observation period. In all other groups metabolic rate was highest on day one after seeding and decreased over the observation period most of all in the cultures seeded on matrices generated from protein separated by means of AS. In those cultures metabolic rate tend to zero after three days (Table 3).

Fluorescence staining of seeded matrices revealed a 3-dimensional arrangement of the LOSMC in matrices generated from protein isolated by means of ethanol and in a lesser extent on matrices generated by cryoprecipitation. A considerable less 3-dimensional extent was found on matrices generated from protein/ fibrinogen separated by means of AS (Figure 4).

Seeded LOEC formed a confluent layer on Matrices generated from ethanol-precipitated fibrinogen and to a lower extend on matrices generated from cryoprecipitated fibrinogen. On matrices generated from fibrinogen precipitated by means of AS LOEC formed no monolayer but only rare loose cell formations (Figure 5).

Discussion

Fibrin preparations have been found to be an adequate scaffold for seeding procedures in tissue engineering, which can be brought in a desired shape and enhance proliferation of different cell types. Since they can be separated from blood autologous matrices can

Metabolic activity of LOSMC seeded on matrices generated from fibrinogen separated by means of AS-, ethanol- and cryoprecipitation (WST-1)

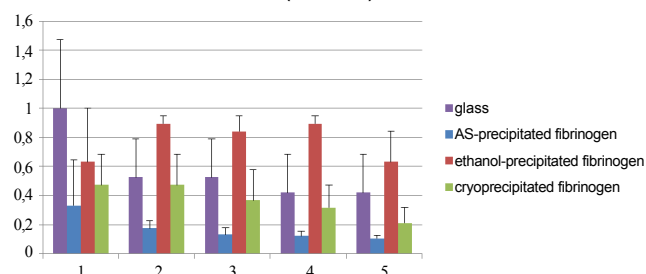


Table 3: Metabolic rate of LOSMC seeded on matrices engineered from proteins separated by cryoprecipitation and addition of ammonium sulphate respectively ethanol. Metabolic rate of LOSMC seeded on gelatin-precoated polystyrene serves as comparison group.

be engineered from a potential later recipient. In this study three methods for the separation of fibrinogen from blood were evaluated. The physiological concentration of fibrinogen in human blood is about 3 g/l [15]. A fibrinogen preparation with that low concentration would be not sufficient for the generation of a stable fibrin matrix. For enrichment proceedings the fibrinogen must precipitated from the plasma and preferably completely dissolved in an adequate solvent. Although other plasma-proteins are precipitated as well and fibrinogen

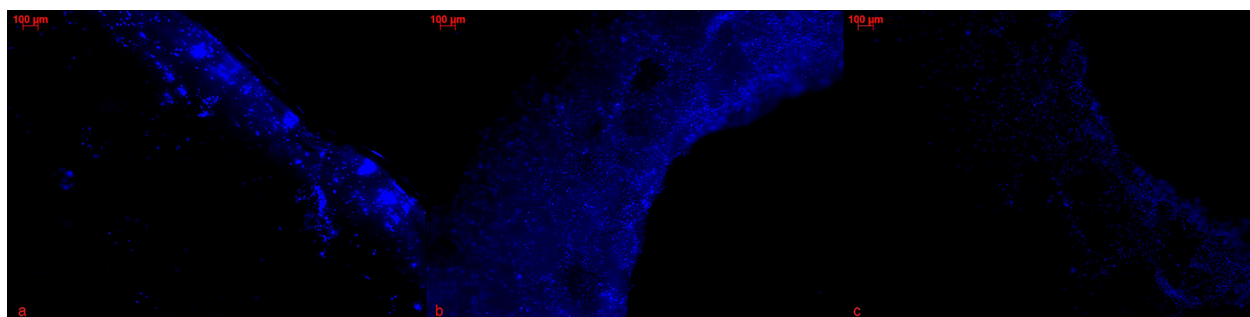


Figure 4: This figure shows cross sections of matrices generated from protein/ fibrinogen-precipitation separated by means of AS (a), ethanol (b) and cryoprecipitation (c). Matrices were seeded with LOSMC five days before. Staining was done with DAPI (magnification 100x): a) matrix generated from cryoprecipitated fibrinogen, b) ethanol-precipitated fibrinogen, c) generated from AS-precipitated fibrinogen.

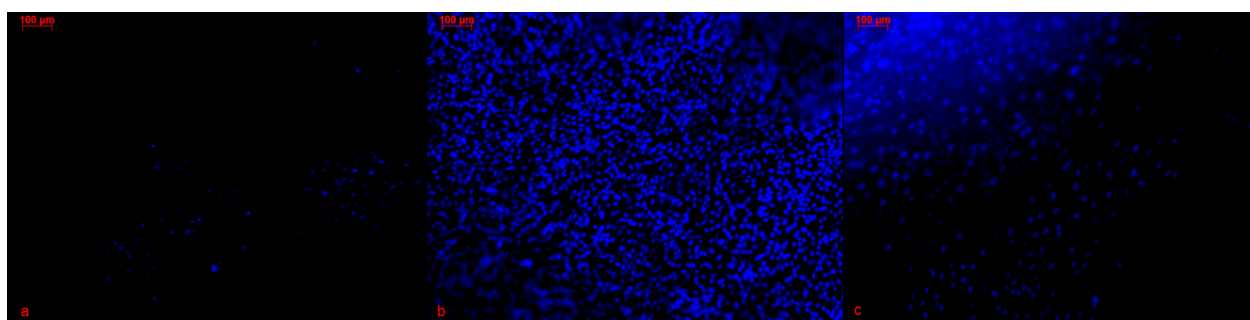


Figure 5: LOEC seeded on fibrin matrices (matrix generated from ethanol-precipitated (a), cryoprecipitated (b) and AS-precipitated (c) fibrinogen). Cells were stained with DAPI. View on the seeded surface of the matrices. In contrast to both others on the matrices generated from ethanol-precipitated fibrinogen LOEC formed a confluent monolayer.

is only a part of the precipitated protein each preparation obtained in this study was clottable and matrices were successfully generated.

A high yield of clottable protein / fibrinogen is of course an advantage, thus the amount of blood needed especially in an autologous approach can be minimized. The largest amount of clottable protein was obtained by means of AS, whereas the least quantity was obtained by cryoprecipitation. However, denaturation and premature clotting complicate the generation of a clottable fibrinogen preparation by addition of AS and ethanol. Best properties concerning the handling of the preparation were found for cryoprecipitation resulting in the highest concentrations of fibrinogen in the final preparation.

In many areas of tissue engineering particularly in the generation of cardiovascular implants a high mechanical strength of the supporting structure is mandatory. The best mechanical properties were exhibited by the matrices from cryoprecipitated fibrinogen. Corresponding to the better mechanical properties a more complex structure with a network of fibrils was found in the matrices generated from cryoprecipitated fibrinogen, whereas the structure of the matrices from the other fibrinogen preparations was less complex.

We assume a denaturation of fibrinogen by the addition of ethanol and AS being responsible for an insufficient cross-linking of fibrin, which is expressed in the lower stability and significantly less complex structure of the corresponding matrices. The relatively high concentrations of fibrinogen measured in this preparation suggest that denatured fibrinogen is detected by the used assay as well. In order to exclude a potential deficit of factor XIII in the preparations, it was added to the thrombin component [16]. The presence of factor XIII has been shown to significantly increase the tensile strengths of fibrin preparations and improve the in-vivo hemostatic efficacy by inducing the crosslink of fibrin chains by covalent bonds [17-19]. Factor XIII is present in preparations generated by cryoprecipitations as well as preparations by means of chemical agents [20,21]. However, addition of factor XIII did not increase mechanical properties or the structure of fibrin matrices corroborating the hypothesis of denaturation of the fibrinogen precipitated by means of ethanol or AS.

Beside adequate handiness and sufficient mechanical stability in distinct applications a carrier is needed for cell application which provides a three-dimensional space and enhances proliferation of seeded cells. A similar application is the seeding of cells on synthetic materials to obtain an endothelialization before implantation with contact to the bloodstream. Endothelial cells need a suitable matrix for spreading and proliferation [22,23]. On matrices which were generated from ethanol-precipitated fibrinogen a higher proliferation of seeded LOSMC resulting in widespread three-dimensional arrangement of the cells was found. Furthermore, LOEC seeded on these matrices formed a confluent monolayer on the surface. The corresponding experiments with cryoprecipitated fibrinogen showed slightly inferior results, while cells on matrices from AS-precipitated fibrinogen showed marginal proliferation. It was shown that cells show a higher proliferation when seeded on less structured matrices according to the matrices from ethanol-precipitated fibrinogen which show a considerable less structured composition than matrices from cryoprecipitated fibrinogen [24]. In contrast matrices generated from AS-precipitated fibrinogen had a similar composition as ethanol-precipitated but there was only marginal proliferation of seeded cells on those matrices. We assume a toxic side effect of AS on the seeded cells.

In conclusion fibrinogen preparations can easily be separated from heparinized blood. Depending on the type of separation method fibrinogen preparations are obtained from which matrices with different properties can be engineered depending on the desired application.

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